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MINI-REVIEW

Tanneke den Blaauwen · Arnold J. M. Driessen

Sec-dependent preprotein translocation in bacteria

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Abstract Translocation of precursor proteins across the cytoplasmic membrane in bacteria is mediated by a multi-subunit protein complex termed translocase, which consists of the integral membrane heterotrimer SecYEG and the peripheral homodimeric ATPase SecA. Preproteins are bound by the cytosolic molecular chaperone SecB and targeted in a complex with SecA to the translocation site at the cytoplasmic membrane. This interaction with SecYEG allows the SecA/preprotein complex to insert into the membrane by binding of ATP to the high affinity nucleotide binding site of SecA. At that stage, presumably recognition and proofreading of the signal sequence occurs. Hydrolysis of ATP causes the release of the preprotein in the translocation channel and drives the withdrawal of SecA from the membrane-integrated state. Hydrolysis of ATP at the low-affinity nucleotide binding site of SecA converts the protein into a compact conformational state and releases it from the membrane. In the absence of the proton motive force, SecA is able to complete the translocation stepwise by multiple nucleotide modulated cycles.

Key words *Escherichia coli* · ATPase · Energetics · Membrane protein · Protein folding · Sec Proteins · Transport

Proteins designed for transport across the cytoplasmic membrane of *Escherichia coli* and other bacteria are synthesized with a cleavable signal peptide at the amino terminus. This signal sequence is recognized by the translocation machinery and acts as the targeting signal that guides preproteins to the translocase at the cytoplasmic

membrane. The translocase is a multi-subunit protein complex with the integral membrane subunits SecY, SecE, and SecG, and with SecA as the dissociable peripheral subunit. These subunits have been found in all prokaryotes studied thus far and also in eukaryotic organelles evolved from bacteria, such as the thylakoidal membrane of the plant chloroplast (Yuan et al. 1994). Preproteins can be bound as nascent chains at the ribosome by the cytosolic chaperone SecB. SecB promotes the interaction of the preprotein with SecA to form a ternary complex. SecA is an ATPase that exists both in a cytosolic and membrane-bound form. At the membrane surface, SecA interacts with negatively charged phospholipids and with the integral subunits SecY, SecE, and SecG of the translocase. The translocation of a preprotein across the cytoplasmic membrane is an energy-requiring event driven by the hydrolysis of ATP and the proton motive force (Δp). Translocation is initiated by the ATP-dependent co-insertion of SecA and the preprotein in the translocase. Interaction of SecA with SecYEG stimulates the ATPase activity of SecA, and this event dissociates the SecA-preprotein complex and allows withdrawal of SecA. The Δp drives the completion of the preprotein translocation. However, in the absence of a Δp , multiple cycles of nucleotide-modulated SecA insertion and withdrawal can also drive the complete translocation. Other proteins like SecD and SecF are thought to function in the later stages of translocation, such as in correct folding of the protein. During translocation, the signal sequence is cleaved by a membrane-bound signal peptidase. In this mini-review, we summarize the data and speculate on the molecular mechanism of the initiation of the preprotein translocation. For more comprehensive reviews and detailed discussion on intermediate stages of translocation and the role of signal recognition particle, see Wickner et al. (1991) and Driessen (1994).

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SecB: a protein traffic-jam aid?

SecB is the only molecular chaperone found thus far that is dedicated to the Sec-dependent transport of proteins. It

Fig. 1 Schematic overview of the amino acid sequence of *Escherichia coli* SecA and the regions involved in ATP, preprotein, membrane, and SecB interaction. The amino acids that when mutated affect the function of SecA are indicated. Az^r confers the protein azide resistant; Az^{ss} confers the protein super sensitive for azide

enhances the translocation of precursors of outer membrane proteins and some periplasmic proteins (Kumamoto and Francetić 1993; Powers and Randall 1995). SecB binds the preprotein as a nascent chain when it emerges from the ribosome (Kumamoto and Gannon 1988) and prevents premature folding (Liu et al. 1989) and aggregation due to hydrophobic interactions (Lecker et al. 1990). SecB targets the preprotein to the SecA subunit of the translocase (Hartl et al. 1990). SecB is a homotetrameric protein of 15-kDa subunits (Watanabe and Blobel 1989) that forms a stoichiometric complex with the mature domain of the preprotein (Lecker et al. 1989; Hartl et al. 1990). SecB appears to be designed to interact with unfolded polypeptide segments at high rates, but with low specificity (Fekkes et al. 1995). The signal peptide is generally not involved in binding (Randall and Hardy 1995), but is thought to delay stable folding of the preprotein (Park et al. 1988) and to enhance the affinity for the SecB/SecA interaction (Hartl et al. 1990; Breukink et al. 1995). SecB is only indispensable for protein translocation at high growth rates (Randall and Hardy 1995) or when translocation is impaired by mutations causing a decrease in the translocation efficiency (Derman et al. 1993; Flower et al. 1994). This suggests that SecB is essential when preproteins queue for translocation. Both membrane-bound and soluble SecA bind the preprotein/SecB complex through direct recognition of SecB and of the signal sequence and the mature domain of the preprotein (Hartl et al. 1990; Hoffschulte et al. 1994). The carboxy-terminal 70 amino acids of SecA are essential for protein translocation, and removal of these residues abolishes the property of SecA to cause aggregation of negatively charged phosphatidylglycerol vesicles (Breukink et al. 1995). This phenomenon is inhibited by SecB, suggesting the presence of a SecB binding site at the carboxyl terminus of SecA (Fig. 1).

Nucleotide binding sites of SecA

SecA is an essential component of the general translocation pathway in *E. coli* and has been found in a wide vari-

ety of gram-positive and gram-negative bacteria, primitive algae and cyanobacteria, and in higher plants. SecA in *Bacillus subtilis* and *E. coli* share an overall homology of 65% (Overhoff et al. 1991; Sadaie et al. 1991) and have been subject to intensive studies that show that their function is very similar. SecA, a 102-kDa protein (Schmidt et al. 1988), is functional as a dimer (Akita et al. 1991; Driessen 1993).

SecA has two essential nucleotide binding sites (NBS) (Matsuyama et al. 1990b; Klose et al. 1993; Mitchell and Oliver 1994): a high-affinity binding site (K_D 0.13 μ M) confined to the amino-terminal domain of the protein (NBS-I), and a low-affinity binding site (K_D 340 μ M) located at two-thirds of the protein sequence of 901 amino acids (NBS-II) (Mitchell and Oliver 1994; Van der Wolk et al. 1993, 1995) (Fig. 1). Residues 102–109 of SecA exhibit the amino acid sequence motif, G(X₄)GKT (the Walker A motif), characteristic of a major class of nucleotide binding sites (Walker et al. 1982). Mutations in SecA leading to substitutions at the invariable Lys of NBS-I block the translocation activity of SecA, consistent with this sequence being part of the phosphate binding site (Van der Wolk et al. 1993; Mitchell and Oliver 1994). These mutations interfere with the release of SecA from the membrane (Van der Wolk et al. 1993), suggesting a coupling between translocation and the temporal insertion/withdrawal of SecA into the membrane (Economou and Wickner 1994). ATP hydrolysis at NBS-I, therefore, appears to be essential for the withdrawal of SecA. The second Walker motif (B-motif), hXXhhD, that in combination with the A motif completes the Mg²⁺-phosphate protein interaction, is found in SecA at residues 205–209. This B motif is atypical as it is repeated at amino acid residues 211–215. Both aspartate residues of the duplicated motif are needed for the coordination shell of the Mg²⁺-ion and are indispensable for SecA function in vivo and in vitro (Mitchell and Oliver 1994; Van der Wolk et al. 1995). Residues 503–511 contain the Walker A motif of NBS-II. Mutations leading to substitutions at Arg509 block the translocation activity of SecA (Mitchell and Oliver 1994). This mutant is blocked in translocation ATPase activity, i.e., in the stimulation of the ATPase activ-

ity by preproteins when SecA is bound at the SecYEG protein, and binds ADP only in NBS-I. The amino acid region 631–653 has been tentatively assigned as the second Walker B motif, but its identity as such has not yet been confirmed. Both NBS-I and NBS-II are indispensable for the translocation activity of SecA. In contrast to their ability to bind ATP, they appear to function in a cooperative manner.

The effects of ATP- and ADP binding on the conformational stability of SecA have been studied by differential scanning calorimetry. SecA unfolds thermally as a two-domain protein of approximately equal size (T. Den Blaauwen, unpublished results). Saturation of NBS-I by ADP increases the stability of the protein and promotes interaction between the domains. Saturation of NBS-II also further pronounces these effects and dramatically increases the compactness of the protein. This conformation of SecA could correspond to the membrane-withdrawn state as these are the conditions under which SecA preferentially associates with the membrane surface (Breukink et al. 1992). Saturation of NBS-I with the non-hydrolyzable ATP analog AMP-PNP, does not stabilize SecA at all (T. Den Blaauwen, unpublished results). Under these conditions, SecA tends to penetrate into the membrane (Breukink et al. 1992). The ATP-bound and membrane-inserted state of SecA could, therefore, correspond to a more extended conformation. Addition of apyrase to deplete the available ATP in an ongoing *in vitro* translocation reaction immediately arrests this reaction, presumably by inhibiting the withdrawal of SecA (Economou and Wickner 1994). Translocation can be resumed after isolation of the vesicles and addition of ATP. This suggests that hydrolysis by both NBS-I and NBS-II is needed for a successful withdrawal of SecA.

Deletion of 66 carboxy-terminal amino acids or replacement of some of the cysteine residues of SecA by serine residues increases the level of ATP hydrolysis and impairs protein translocation (Rajapandi and Oliver 1994). This suggests that the carboxy-terminal region of SecA facilitates the coupling of its ATPase activity to cycles of protein translocation (Rajapandi and Oliver 1994). Site-directed tryptophan fluorescence spectroscopy studies demonstrate that the binding of ADP at NBS-I (Fig. 1) changes the conformation of the carboxyl terminus of SecA. This again suggests that communication between NBS-I and a carboxy-terminal domain of SecA is essential for SecA function (T. Den Blaauwen, unpublished results) (See Membrane association and topology of SecA).

Signal sequence proofreading function of SecA

Sodium azide is an inhibitor of the Sec-dependent protein translocation pathway in *E. coli* (Oliver et al. 1990) and in *B. subtilis* (Nakane et al. 1995). Protein translocation by sodium azide is inhibited by blocking of the translocation ATPase activity of the SecA protein. Azide-resistant mutants show an elevated translocation ATPase activity. The majority of the azide-resistant mutants are found to have a

replacement of amino acid 134 in *E. coli* (Fortin et al. 1990; Huie and Silhavy 1995) and amino acid 128 in *B. subtilis* (Nakane et al. 1995). These residues are found in the region between the Walker A and B site of NBS-I (Fig. 1). An accompanying effect of these *azi* mutations is that they, like the *prlD* mutations, enable the translocation of proteins with a defective signal sequence (Huie and Silhavy 1995). The *prl* (for protein localization) class of mutants are all isolated as suppressors of signal sequence mutations and have been found in the SecA (*prlD*) (Fikes and Bassford 1989; Schatz et al. 1991; Huie and Silhavy 1995), SecY (*prlA*) (Derman et al. 1993; Flower et al. 1994), and SecE (*prlG*) (Flower et al. 1994) subunits of the translocase (for a review, see Schatz and Beckwith 1990). It has been proposed that *prl* suppressors function not by restoring the recognition of altered signal sequences, but rather by preventing the rejection of defective preproteins from the export pathway (Osborne and Silhavy 1993). For the *prlD* mutants, this could be achieved by a slow down of the ATPase activity, thereby prolonging the presentation of the preprotein to the translocase (Huie and Silhavy 1995). According to this hypothesis, SecA, SecY, and SecE would have a proofreading activity. Another class of *prlD* mutants in SecA are the azide super-sensitive mutants (*Az^{ss}*), which are clustered in the vicinity of the Walker A site of NBS-II (Fig. 1) (Huie and Silhavy 1995). The signal-sequence-defect-suppressing phenotype and the *Az^{ss}* phenotype caused by these *prlD* mutants are dominant in diploid analysis (Huie and Silhavy 1995). A possible explanation could be that in the presence of azide, these mutants are unable to hydrolyze ATP once they have inserted in the membrane. Thus, they would block access to translocation channels for the *in trans* wild-type SecA. *Az^r* is also conferred by suppressor mutations within or in the vicinity of either subsite of NBS-II, rather than in the intervening sequence. The suppressor phenotype of these mutants is either rather weak or unknown, indicating that this ATP-binding site might not be essential for the signal sequence proofreading.

SecA interaction with preproteins

Preproteins weakly associate with SecA in solution, but associate with high affinity with membrane-associated SecA. SecB promotes this interaction by binding SecA (see above) and prevents the premature release of the preprotein from SecA (A. J. M. Driessen, unpublished results). Biochemical evidence demonstrates that SecA interacts with preprotein through recognition of the positive charge at the amino terminus of the signal peptide (Akita et al. 1989) and through binding of the mature domain of the preprotein (Cunningham and Wickner 1989; Lill et al. 1990; Akita et al. 1991; Kumamoto 1991). The mutated SecA protein SecA51^{TS} has been immunoprecipitated under non-denaturing conditions from the cytosolic fraction of cells grown at the non-permissive temperature. Analysis by two-dimensional SDS electrophoresis reveals that incomplete nascent and fully elongated maltose-binding

protein co-immunoprecipitate with SecA51^{TS} (Chun and Randall 1994). These results suggest that both cytosolic and membrane-associated SecA function in preprotein translocation. Cross-linking studies with a large variety of amino-terminal and carboxy-terminal SecA peptides suggest that the domain in SecA that interacts with the preprotein is located between amino acid residues 267 and 340 (Kimura et al. 1991) (Fig. 1). The ADP-bound form of SecA has a higher affinity for the preprotein than the ATP-bound form (Shinkai et al. 1991). In solution, preprotein binding releases ADP from NBS-I of SecA and stimulates ADP/ATP exchange at this site (Shinkai et al. 1991). ATP hydrolysis dissociates the SecA/preprotein complex bound at the SecYEG protein (Schiebel et al. 1991).

Membrane association and topology of SecA

SecA is able to bind to protein-free liposomes provided that they contain negatively charged phospholipids (Lill et al. 1990; Kusters et al. 1991; Ulbrandt et al. 1992; Keller et al. 1995). This binding stimulates SecA for ATP hydrolysis, but in the absence of SecYEG and translocation-competent preproteins, the ATPase activity of SecA is thermal labile. This activity is termed "lipid-ATPase", whereas the ATPase activity of SecA in the presence of all interacting partners is termed "translocation-ATPase" (Lill et al. 1989). The presence of SecYEG is essential for a productive interaction of SecA with the membrane (Lill et al. 1989; Joly and Wickner 1993). Cross-linking studies have shown that preprotein segments that are initially associated with SecA move into contact with SecY as they traverse the membrane, yet they never lose their contact with SecA until they have emerged on the periplasmic face of the membrane (Joly and Wickner 1993). It has been noted that 20–30 amino acid residues of the preprotein are driven across the membrane when non-hydrolysable ATP analogs bind to the translocase bearing a defined translocation intermediate (Schiebel et al. 1991; Arkowitz et al. 1993). This may be the amount of the preprotein that is carried into and partially across the membrane by the movement of SecA.

Proteoliposomes reconstituted with a detergent-soluble fraction of urea-stripped inside-out (ISO) vesicles (i.e., vesicles containing urea-extraction-resistant SecA), have been shown to be capable of protein translocation, whereas immunologically SecA-depleted proteoliposomes are inactive (Watanabe and Blobel 1993). This shows that at least part of the active SecA population is in a membrane-integral form. Proteolysis studies with rightside-out (RSO) membrane vesicles of *E. coli* suggest that SecA penetrates the cytoplasmic membrane in its membrane-bound form, exposing domains to the periplasm (Kim et al. 1994). A 30-kDa SecA fragment has been found to be resistant to protease K digestion of ISO membrane vesicles during *in vitro* preprotein translocation in the presence of ATP (Economou and Wickner 1994). Even in the absence of preprotein translocation, part of the SecA molecule is in-

temporarily associated with the cytoplasmic membrane (Cabelli et al. 1991) and can only be partly removed by treating the vesicles with urea (Cunningham et al. 1989). In ISO membrane vesicles, a 35-kDa membrane-interacting domain of SecA has been found that is relatively trypsin insensitive. Based on monoclonal antibody mapping, this fragment has been shown to consist of approximately amino acid residues 1–310 (T. Den Blaauwen, unpublished results). This fragment may overlap with the 30-kDa membrane-inserting domain (Economou and Wickner 1994) and the SecA peptide (1–239) that is found entirely associated with the membrane (Cabelli et al. 1991). The latter has been shown to be able to complement the *secA51(TS)* mutant, which has an altered amino acid residue 43 (Schmidt et al. 1988). A second, carboxy-terminal domain of SecA consisting of at least part of the amino acid sequence 850 up to 901 has been found to be embedded in the membrane or in the translocase and to be accessible from the periplasmic face of the membrane in RSO membrane vesicles (T. Den Blaauwen, unpublished results). This implies that the carboxy-terminal region of SecA penetrates through the entire membrane. In *E. coli* SecA, the strongly positively charged carboxy-terminal motif RNXPCCGSGKK ϕ KXCXG, which is present in all bacterial SecA molecules found thus far, is preceded by a hydrophobic sequence. It is possible that the positively charged sequence associates with the negatively charged phospholipids, which subsequently induces the insertion of the hydrophobic region in the membrane. SecB binds the carboxyl terminus, which makes it conceivable that association of this sequence with the membrane and subsequent insertion into the membrane of the carboxyl terminus dislocates SecB from SecA at the initiation of the translocation.

The integral membrane subunits of the translocase

The integral membrane subunits SecY, SecE, and SecG of the translocase copurify as a heterotrimeric complex when a cytoplasmic membrane detergent extract is fractionated. Furthermore, the complex can be immunoprecipitated with anti-SecY, anti-SecE, and anti-SecG antisera (Brundage et al. 1990, 1992). Overproduced SecY is extremely labile without the presence of overproduced SecE, suggesting that SecY and SecE physically interact (Matsuyama et al. 1990a). Recent studies have shown that SecY, SecE, and SecG assemble *in vivo* as a stable heterotrimeric complex (Joly et al. 1994). SecY is a 49-kDa polypeptide with ten putative transmembrane helices (TMH) (Ito 1992), SecE is a 14-kDa polypeptide that contains three TMHs in *E. coli* (Fig. 2) and in many other bacteria only one TMH (Schatz et al. 1989). The first two TMHs of *E. coli* SecE (residues 7–78) are dispensable for protein translocation (Schatz et al. 1991). Intriguingly, SecY and SecE are homologous to the α - and γ -subunits of the heterotrimeric Sec61 complex involved in preprotein translocation into the yeast and mammalian endoplasmic reticulum (Görlich and Rapoport 1993; Hartmann et al. 1994). These proteins

Fig.2 Schematic overview of the putative topology of *Escherichia coli* SecY and SecE. Indicated are clusters of mutations involved in SecY-SecE interaction (*filled circles*) and clusters of strong signal peptide defects suppressing mutants (*open squares*)

Fig.3 Hypothetical model for the ATP-dependent initiation of preprotein translocation by the Sec-dependent protein translocation pathway. *Steps: I* Signal peptide proofreading, *II* SecA membrane insertion and preprotein translocation, *III* SecA withdrawal and release of preprotein, and *IV* proton-motive-force-driven completion of translocation

may thus have similar roles in the bacterial and mammalian systems. Several pairs of *prlA* (SecY) and *prlG* (SecE) alleles that exhibit synthetic defects based on complementation screening have been found (Flower et al. 1995). These pairs suggest an interaction between the first amino-terminal periplasmic loop of SecY and the carboxy-terminal loop of SecE, between TMH7 of SecY and TMH3 of SecE, and between TMH10 of SecY and TMH3 of SecE (Flower et al. 1995; filled circles in Fig. 2). None of these *prl* mutations belong to the class of strong signal peptide defect suppressors, an indication that the SecY-SecE-interacting amino acid residues might not necessarily correspond with the residues that are involved in the “proofreading” activity. The mutated protein SecY24 (G240I) or SecY with a deletion in the cytoplasmic loop 4 can no longer be co-immunopurified with SecE, indicating that this loop presumably also interacts with SecE (Baba et al. 1994). The vast majority of known strong signal-sequence-defect-suppressing *prl* mutations map to *prlA* (SecY), whereas the weaker suppressing mutations map to *prlG* (SecE) and to *prlD* (SecA) (Flower et al. 1994; Huie and Silhavy 1995) (Figs. 1 and 2). Again, most *prl* mutations cluster in the domains of SecY and SecE described above, albeit a larger variety of residues seem to have a high frequency of amino acid exchange

(Flower et al. 1994; open squares in Fig. 2). The majority of the *prlA* mutations are found in TMH7 of SecY, suggesting that this helix is intimately involved in the proofreading process. Cross-linking studies of *in vitro* translocation of proOmpA (the precursor of outer membrane protein A) with unique cysteine residues within the mature part of the preprotein (amino acid residues 245–315) reveal that these cysteines could only be cross-linked to SecA and SecY, but not with SecE, SecG, or phospholipids (Joly and Wickner 1993), indicating that at least SecA and SecY are intimately associated with the mature part of the preprotein during translocation.

SecG is a 11.4-kDa polypeptide with two putative TMHs and a large carboxy-terminal cytoplasmic domain (Nishiyama et al. 1993), which is essential for cell growth and protein translocation at low temperatures, but is dispensable under non-restrictive conditions (Nishiyama et al. 1994). SecG function can be compensated for by an increase in the level of acidic phospholipids, which are essential for the association of SecA with the membrane (Kontinen and Tokuda 1995). Therefore, it is thought that SecG might facilitate the insertion of SecA at the translocase, especially at low temperatures when the membrane fluidity is low (Kontinen and Tokuda 1995).

A hypothetical model for the initiation of preprotein translocation

A tentative model for the events leading to the initiation of translocation are schematically represented in Fig. 3. The signal sequence of a preprotein/SecB complex binds to the ADP-bound compact state of SecA. The tertiary complex traverses to or along the membrane until it encounters the integral membrane translocase subunits. Interaction of SecA with these subunits stimulates the protein for ADP/ATP exchange, and the binding of ATP to SecA causes insertion of the carboxyl terminus of SecA into the membrane and the release of bound SecB. Membrane insertion of SecA makes the signal peptide domain of the bound preprotein accessible to SecY and SecE, and proofreading takes place (Step I). The membrane-inserted signal sequence may enter the translocation channel laterally, as suggested for the translocase of mammalian endoplasmic reticulum (Martoglio et al. 1995). If SecY and SecE are able to recognize and to bind the signal sequence, the translocase channel opens, and concomitantly allows access of the amino-terminal domain of SecA with the mature part of the preprotein (Step II). This stimulates the ATPase activity of SecA at the high-affinity nucleotide binding site, causing SecA to release the preprotein. Subsequent ATP hydrolysis at the low-affinity nucleotide binding site induces the compact conformation of the ADP-bound state and causes withdrawal of SecA from the membrane (Step III). In the presence of the proton motive force (Step IV), translocation will be completed without further involvement of SecA (Schiebel et al. 1991; Driessen 1992). Alternatively, SecA may bind the mature part of the preprotein and re-enter the ATP-driven translocation until completion (Schiebel et al. 1991).

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